Chapter SEVEN

Summary and general discussion

The first 1000 days is a period of very rapid growth and development during which the infant needs a high supply of nutrients. Nutrients and especially lipids provide energy for growth but are also important factors for brain development, contributing to structure, volume and functional neurocircuitry development. Before birth, infants get most of the necessary nutrients via the mother, but after birth the nutritional supply of brain building blocks are provided via the maternal milk. Human milk (HM) is considered the best nutrition that can be provided to infants, containing all nutrients that the infant brain needs. When breastfeeding is not (or no longer) available Infant Milk Formula (IMF) provides an alternative source of nutrition.

There are many studies that show that breastfeeding (duration) is positively associated with infant neurodevelopmental outcomes compared to formula feeding. There are many differences between breastfeeding and formula feeding that may contribute to this effect, including behavioural aspects (e.g. mother child bonding) as well as compositional differences between HM and formula, such as the presence of hormones, growth factors and immune factors in HM. Although energy content, gross macro and even micro nutrient composition of HM and IMF may be similar, many differences in nutritional quality remain, including the quality of dietary lipids. The dietary fatty acid composition is an important aspect of dietary lipid quality in milk. Fatty acid composition is constant in IMF, whereas it quite variable in HM, reflecting the diet of the mother and perhaps the changing needs of the developing infant brain. Another aspect of dietary lipid quality that differs between IMF and HM is the physical appearance of lipid globules (i.e. the supramolecular lipid structure). Mammalian milk contains large lipid droplets that are surrounded by a biological membrane as a result of the normal physiological lactation process, whereas lipid droplets in standard IMF are much smaller and are not surrounded by a membrane due to processing to create a reproducible, stable and safe solution. We investigated the contribution of these two quality aspects of the quality of dietary lipid supply in early life to brain development and function using mice models. We focused on a number of different aspects of brain development, including neuronal membrane lipid composition, structural development of networks, and functional aspects (behaviour, cognition), taking into account both acute and long term effects, and the effects of different environmental conditions that may influence nutrient metabolism.

Summary of the results in Chapters 2-6

Omega (n) 3 long-chain polyunsaturated fatty acids (LCPUFA's) are important for brain development and accumulate rapidly in the brain during the first 1000 days. Brain n-3 LCPUFA accretion depends on the dietary supply of both n-3 and n-6 LCPUFA's and their C18 precursors: linoleic acid (LA; C18:2n-6) and alpha-linolenic acid (ALA; C18:3n-3), that are referred to as essential fatty acids (EFA). The contemporary increase in dietary intake of LA and decrease in n-3 LCPUFA intake in the population is also reflected in human milk polyunsaturated fatty acid (PUFA, i.e. both LCPUFA and EFA) composition. As human milk is the sole source of nutrition for newborn infants this could also impact infant brain fatty acid accretion patterns. To study the effects of maternal dietary FA composition on milk FA composition and subsequent accumulation of n-3 LCPUFA and other FA in the brain of offspring during the lactation period, lactating female mice with litters were exposed, from 2 days after birth of the litter onwards, to a control diet or either of two experimental diets with a reduced n-6/n-3 PUFA ratio. In one of the experimental diets the reduced n-6/n-3 PUFA ratio was achieved by 50% reduction of LA, and in the other experimental diet the reduced n-6/n-3 PUFA ratio was accomplished by increased content of n-3 LCPUFA's, including eicosapentaenoic acid (EPA; C20:5n-3), docosapentaenoic acid (n-3 DPA, 22:5n-3) and docosahexaenoic acid (DHA, C22:6n-3). As described in **Chapter 2**, we found that the maternal experimental diets resulted in reduced levels of LA and increased levels of preformed n-3 LCPUFA content in the maternal milk respectively compared to milk of control dams. Both these interventions, however, increased the accumulation of n-3 LCPUFA's including DHA and reduced the content of n-6 LCPUFA's in the offspring brain. In case of the low-LA diet group, the higher brain n-3 LCPUFA's were originating from n-3 LCPUFAs newly synthesized by the pups, which was possible due to the low supply of the competing LA in the milk. In the n-3 LCPUFA diet group, the preformed n-3 LCPUFA's in milk were the main source of the n-3 LCPUFA's accumulating in the offspring brain. These data suggest that for humans exposed to the generally excessive LA levels in our modern Western diet, the dietary recommendations to pregnant and lactating women should include an advice that will facilitate reduced LA intakes next to higher n-3 LCPUFA.

Next, in **Chapter 3** we used the same mouse model and found in the offspring that both the n-3 LCPUFA and low-LA diet also resulted in a different structural formation (i.e. axonal density) of the hypothalamic circuitry that controls energy balance. The structural changes were already present at one week after weaning, and were sustained into adulthood, 8 weeks after termination of the experimental diets and after being switched to a moderate Western Style Diet (WSD; 21 En% fat of which 11 En% lard and 0.2 En% cholesterol). The structural alterations of the hypothalamic circuitry suggest a different energy balance regulation throughout life and may be linked to the resistance to adult diet- induced obesity that was previously reported for these experimental diets (1, 2). Maturation of the hypothalamus in mice continues during the lactation period, and it is well known that within this restricted time period, circulating leptin levels in the offspring follow a distinctive surge, which plays a crucial role in mediating the outgrowth of axons from hypothalamic neurons forming the hypothalamic circuitry. As circulating leptin levels are responsive to nutrient supply,

it was hypothesized that leptin was the factor mediating the effects of dietary fatty acid composition on hypothalamic circuitry maturation in the current study. However, in contrast to our hypothesis the postnatal leptin surge remained unaffected by the experimental diets. This suggests that other, perhaps yet unidentified mechanisms that are responsive to dietary n-6 and n-3 PUFA can also contribute to the structural development of the hypothalamic circuitry. Besides affecting hypothalamic structures; also the development of other brain structures involved in for instance cognitive functioning could be influenced by dietary lipids in early postnatal life. Indeed, alterations in the dietary supply of n-3 and n-6 PUFA early in life affect cognitive function (3).

In **Chapter 4** we describe that another aspect of lipid quality, i.e. the physical properties of lipid globules, may also contribute to brain development. Starting after 2 weeks of normal lactation, juvenile mouse pups (and dam) were exposed to a rodent concept diet in which the lipid fraction existed of large droplets that were coated by a phospholipid (PL) layer (concept diet), resembling more closely the supramolecular structure of lipids in mammalian milk. Mice fed this diet showed increased attention and working memory performance compared to mice fed a control diet with small lipid droplets and no PL-coating, while spatial reference memory remained unaffected. The increased recognition memory was sustained into adulthood and the effects could not be explained by alteration in brain PL composition. Increased cognitive performance due to the experimental diet was observed specifically in test that involved novelty exposure, which induces a state of mild arousal in mice. It was therefore hypothesized that the observed effects of concept diet were mediated by a different development and or functioning of brain circuits that modulate overall cognitive function during arousal.

The experiment described in **Chapter 4** was conducted using mice that were housed individually from weaning onwards. Mice, however, are social species by nature and deprivation of social contact with conspecifics can be stressful. In addition, the deprivation of social thermoregulation, a natural behaviour for mice, can alter energy metabolism. In particular early in life social and metabolic stress can interfere with normal growth and development and the social housing situation after weaning may thereby affect the later in life functioning and phenotype of the mouse. It is known that social isolation stress in rodents during adolescence impairs structural development and functioning of brain areas involved in anxiety, reward and cognitive functioning (4, 5). Besides stress induced alteration in neurochemistry however, growth trajectories and metabolic status are closely related to brain development and function and it is therefore conceivable that some of the known neurodevelopmental effects of social isolation rearing may be mediated by the metabolic adaptations to individual housing. Moreover, it can also be hypothesized that effects of early life nutrition on brain functions (whether or not mediated by metabolic adaptations), such as the effects of supramolecular lipid structure on cognitive function as described in **Chapter 4**, could be affected by the social housing situation of the mice. This idea is further supported by the observation that the programming capacity of early life diet with altered supramolecular lipid structure on adult body fat accumulation appeared to be different depending on the social housing situation of the mice, as described in the **Intermezzo**. Whereas the consequences of social isolation stress on cognitive and behavioural

functioning in rodents are well described in the literature, the effects of individual housing on metabolic phenotype are not. **Chapter 5** reviews the published evidence for effects of individually vs social housing on bodyweight and metabolic outcomes in mice and rats. A meta-analysis indicated that although the published effects on bodyweight were not consistent, a higher food intake and increased adiposity due to individual housing was observed, which suggest that individual housing results in maladaptive metabolic function. Next, in **Chapter 6**, we investigated whether the previously observed (**Chapter 4**) changes in cognitive performance by the altered supramolecular structure of dietary lipid globules were dependent on the housing situation of the mice. Confirming our previously reported results obtained in individual housed animals, the diet with large, PL coated lipids resulted in increased recognition memory during adulthood in both individual and social housed animals. Effects were observed together with augmented growth and bone development during adolescence and reduced adolescent anxiety like behaviour. The effects of diet were however not dependent on the social housing situation. It was hypothesized that the enhanced growth and bone development that were caused by diet contribute to the development and functioning of the brain. Individual housing reduced growth rate but did not reduce brain myelination and cognitive function, which was unexpected. Social isolation is generally believed to be aversive to rodents and is known to lead to various abnormalities in brain development including reduced myelination and impaired cognitive function. It is hypothesized that in the current study, the particular individual housing paradigm that was applied, i.e. individually housed in the cage after weaning but olfactory, auditory and visual contact was still possible, is not as aversive to for male mice as true social isolation and therefore did not lead to altered myelination and cognitive impairments.

Possible mechanisms by which dietary lipid quality influences brain development and functioning

The results described in this thesis show that the quality of dietary lipids, as PUFA composition or supramolecular lipid structure, during critical phases of growth and development can influence the development and long term function of the brain. Effects of dietary lipid quality were observed on neuronal membrane fatty acid composition, the formation of distinctive neuronal circuits and on specific cognitive and emotional functions under various environmental conditions that impact metabolic functioning. Current understanding of the underlying mechanisms by which dietary fatty acid composition and the supramolecular lipid structure can adapt brain development and function include a number of pathways that are specific for the different aspects of dietary lipid quality (i.e. lipid composition, supramolecular lipid structure, bioactive lipid components) although some overlap is likely. Firstly, dietary lipids influence physical growth and functioning of neurons, brain structures and networks through their function as building blocks for the neuronal membranes. Dietary fatty acid composition, the supramolecular structure of lipids, and specific bioactive lipid components can all change the availability and incorporation of these building blocks for the developing brain. Secondly, as incorporated membrane components or as circulating factors,

lipids may be used as neurodevelopmental signals for differentiation and proliferation of neurons and the structural and functional maturation of specific neuronal networks. Third, specific (hormonal and metabolic) factors, nutrients and metabolites that are released by peripheral organs in response to fatty acid composition and or supramolecular structure of lipids can act as neurodevelopmental signals or may be used as building block for the brain. Finally, important for consideration is the contribution of other environmental factors on brain development and function, which may also interact with dietary lipid quality. Some of the proposed mechanisms that may apply to the results in this thesis are described in more detail below.

Building blocks: Dietary PUFA composition influences brain PUFA accumulation

Nearly 60% of the dry-weight of the human brain consists of lipids (6). About 35% of the lipids in the grey matter are LCPUFA's which can be found as structural components of the neuronal membrane (7).The most abundant LCPUFAs in the neuronal membrane are docosahexaenoic acid (DHA, C22:6n-3) and arachidonic acid (ARA, C22:4n-6) which both rapidly accumulate in brain tissue during development (8). These two LCPUFAs contribute to membrane physical properties and have their own unique roles in brain development and function. For example, DHA is critical for various processes important for neuronal growth and development including modulating neural metabolism, differentiation, plasticity, neuroprotection and anti-inflammatory effects (see e.g. (9) for review). ARA is the precursor for specific membrane derived eicosanoids which are important for immunity and immune responses including the regulation of neuroinflammation (10, 11). Other n-3 LCPUFA's in the brain, though present in much lower concentration than DHA, include eicosapentaenoic acid (EPA; C20:5n-3) and docosapentaenoic acid (n-3 DPA, 22:5n-3), that also generate lipid derived mediators that play a role in the inflammatory response (9) and in particular EPA stimulates neurite outgrowth during development (12). DPA from the n-6 family (n-6 DPA; 22:5n-6) is the structural homologue of DHA and typically accumulates in the brain when DHA supply is insufficient (13, 14). This compensatory mechanism ensures that the total brain volume remains the same, however, functionally n-6 DPA does not fulfil the same neurodevelopmental actions as DHA does, which further demonstrates the essentiality of DHA for brain development (12, 15-17). High levels of n-6 DPA are therefore considered disadvantageous. The relevance of sufficient accumulation of n-3 LCPUFA in the brain during the first 1000 days of life for later life brain function including cognitive performance but also in relation to risk for depression/anxiety or other abnormalities is well documented in animal studies (e.g. (18-23)). Besides affecting neural growth and structural development early life, LCPUFA accumulation patterns in the brain have a lasting influence on brain fatty acid composition. In a recent mouse study it was shown that early life introduction of DHA maintains high DHA levels in the brain later in life, even after exposure to a low-DHA diet after weaning (24). Higher n-3 LCPUFA content in the adult brain provides another mechanism by which early life fatty acid accumulation may influence brain function in adulthood. In humans, the direct association between early life brain PUFA composition and later in life behaviour or cognition is much more difficult to demonstrate as this would require the

analysis of brain tissue. Yet, there are several postmortem studies that relate reduced n-3 LCPUFA or increased n-6 LCPUFA concentration in the adult brain with neuropsychiatric disease (25-27). In addition, prospective studies have shown that fetal cord blood DHA levels - which may serve as a proxy for brain PUFA status at birth - are inversely related to internalizing problem behaviour, hyperactivity and inattention in childhood (28, 29), whereas higher levels of n-3 LCPUAFs are associated with better motor performance, (30), higher scores in cognitive functioning (31, 32) and better neurological scores (33). Together, these studies suggest that sufficient accumulation of n-3 LCPUFA in the perinatal developing brain has a long-term impact on brain function and mental health.

The developing brain relies on the plasma pool of LCPUFA's for accumulation. Although the brain is able to generate some DHA by endogenous synthesis from precursor n-3 LCPUFA's in glial cells (34), DHA synthesis in the brain occurs at a much lower rate than the total rate of brain accretion of DHA needed for development (35, 36). This demonstrates the importance of the plasma pools of DHA for brain DHA accumulation. In plasma, DHA is present in two major pools; 1) bound albumin, as non-esterified fatty acid (NEFA-DHA) or lysophosphatidylcholine-esterified (LPC-DHA), or 2) in lipoproteins esterified to e.g. TG, PL, cholesterol, PL or other lysophospholipids. There is no consensus on what lipid form in the plasma pool is preferentially used for accumulation in the brain (37-39), nor are the mechanisms by which these various forms of circulating PUFAs are delivered from plasma to the brain, which involve protein mediated transport and/or passive diffusion, agreed upon (40). Recently, the major facilitator superfamily domain-containing protein 2 (Mfsd2a) was identified as the primary transporter for the transport of DHA and AA in the form of LPC across the blood brain barrier (41). In humans, mutations in the gene encoding this protein impair brain growth and are lethal, which signifies the importance of this type of transport during development (42). However, while it was shown that plasma LPC-DHA is more effectively incorporated in the brain compared to NEFA-DHA (43), other studies show that the total accumulation rate of NEFA-DHA is still higher than LCP-DHA (39). In addition, DHA esterified in lipoproteins is also used as a source for accumulation (44). Regardless, the uptake process from plasma to tissue appears to be non-selective for n-6 and n-3 LCPUFA's and the LCPUFA accumulation of DHA in the brain is therefore dependent on the total and relative levels of both n-3 and n-6 LCPUFAs in the plasma (45, 46). In order to increase brain DHA accumulation it is key to preserve high circulating DHA as well as to prevent excessive levels of n-6 LCPUFA's in plasma. The dietary fatty acid composition plays a central role in this regulation of plasma n-3 and n-6 LCPUFA content, and thereby contributes to the total accumulation of brain n-3 and n-6 LCPUFA's. Circulating n-6 and n-3 LCPUFA's available for brain accumulation can be directly obtained as "preformed" LCPUFA's from dietary lipid sources. As shown in **Chapter 2**, a higher content of preformed DHA in the dam's milk resulted in higher accumulation of DHA in the suckling offspring brain.

Plasma LCPUFA's can also be derived from endogenous synthesis from the dietary derived 18-carbon essential fatty acids (EFAs) (47). The biosynthesis of LCPUFA in the liver and other tissues in the infant involves a series of elongation and desaturation steps by which the precursor of the n-6 family, LA (C18:2 n-6), is converted to arachidonic acid (ARA), and the precursor of the n-3 family, alpha linoleic acid (ALA, C18:3n-3) ALA, to eicosapentaenoic

acid (EPA) and docosahexanoic acid (DHA) (48). LA and ALA use the same set of enzymes and therefore compete with each other for conversion. In **Chapter 2**, it was shown that a reduced content of dietary LA in the milk of suckling mice increased the brain accumulation of DHA and other n-3 LCPUFA's, which is indeed in line with higher endogenous conversion rate of n-3 precursors in the offspring. In human infants, the endogenous synthesis rate of LCPUFAs is limited and insufficient for especially the high demand of DHA (49, 50). In addition, a disproportionally high dietary supply of LA over ALA, as present in the typical human Western-style diet and milk of women consuming this diet (51), results in a high n-6 LCPUFA status. High levels of n-6 LCPUFA in the circulation reduce the relative availability of DHA for uptake from plasma to brain, and inhibit the endogenous synthesis of DHA in the liver, which further reduces the total DHA availability for incorporation and use in the developing brain (48, 52). Thus, optimizing the dietary fatty acid supply to the infant (i.e. increasing preformed n-3 LCPUFA's as well as reducing n-6 LA precursors in (maternal) diet) provides a mechanism by which infant brain n-3 LCPUFA accumulation can be augmented, supporting the high needs of the developing brain.

Building blocks: (Supra)molecular structure of dietary lipids influences brain LCPUFA accumulation

The absolute and relative amounts, and the types (e.g. EFA, preformed) of dietary n-3 and n-6 LCPUFAs directly influence omega 3 and 6 LCPUFA accretion by the brain during development as shown in **Chapter 2**. Another dietary factor that may be of influence on the pattern and or total amount of n-3 and n-6 LCPUFA accumulation in the brain is the molecular structure in which lipids are present in the diet. LCPUFA's in dietary lipid sources can be present as triglycerides (TG) and as PL. In human milk about 85% of the LCPUFAs is in the form of TG and 15% of the LCPUFAs are PL bound (53), while standard IMF contains TG only. The molecular structure of dietary LCPUFAs influences the digestion and absorption kinetics after ingestion, and the subsequent distribution and bioavailability of LCPUFA in the plasma (54, 55). Several studies have shown that PL-bound LCPUFAs, including DHA, are better absorbed by the brain than TG-bound LCPUFA (56-59). The molecular structure of dietary PUFA's may differentially affect their distribution in plasma fatty acid pools (NEFA, LPC, esterified) after digestion and absorption and influence the total and temporal availability for uptake in the brain (35, 55). Regardless of the *plasma* lipid pool, there are several studies that show that *dietary* PL-bound LCPUFA's more effectively target the brain than dietary LCPUFAs supplied as dietary TG (35, 60-63). This extends to the molecular structure of their dietary essential precursors as PL bound ALA was more effective than TG bound ALA in restoring brain DHA levels after perinatal dietary n-3 LCPUFA deficiency in rats (64).

Adding to molecular structure, the *supra*molecular structure of dietary lipids may also influence the bioavailability of PUFA's after ingestion. Mammalian milk has a distinct supramolecular structure as a result of the physiological process by which the fat globules are produced and secreted from the mammary gland cells. Lipid droplet in milk are large, with an average mode diameter between 3 and 5m (65) and consist of a TG core which is

surrounded by the so called milk fat globule membrane (MFGM), a biological membrane composed mainly of PL, cholesterol and some other bioactive components (66). The MFGM is particularly rich in unsaturated fatty acids, although a substantial amount of LCPUFA is also present (67). The lipid droplet size and surface affect absorption and digestion kinetics, thereby influencing the pattern of lipid appearance in the circulation and their bioavailability for organs (54, 68-72). The concept diet that was used in the studies described in **Chaper 4 and 6** contained large lipid droplets that were coated by PL sourced from bovine MFGM, thereby mimicking the supramolecular structure of lipid droplets in mammalian milk (73). An increased bioavailability of LCPUFAs by the different molecular and supramolecular structure of lipids in this diet compared to those in regular IMF (i.e. small lipid droplets without complex surface area) could have amplified brain n-3 LCPUFA accumulation, which could help explain the higher score in specific cognitive tests that were observed in these mice (**Chapter 4, 6**). It was recently shown that IMF with this particular dietary supramolecular lipid structure resulted in a faster postprandial increase in plasma TG and earlier peak NEFA concentrations in adult men (74). Whereas the plasma fatty acid composition was not analysed in that study, preliminary data from a mouse study with the same diet suggests that the bioavailability of especially n-3 LCPUFA's for incorporation in the brain could be increased. In that mouse study, the relative content of (n-3) LCPUFA's in the brain neuronal membranes was found to be higher in specific brain regions important for cognitive processing including hippocampus, prefrontal cortex, and olfactory bulbs following exposure to the diet containing the lipids with more mammalian milk-like supramolecular structure between postnatal day 16 and 42 (*Schipper et al. unpublished data*). The preliminary results of a second mouse study suggest that the increased n-3 LCPUFA incorporation in the developing brain is not merely the result of the addition of PL as ingredient to the diet, as post-weaning exposure to a diet containing large, PL coated lipid droplets was more effective in restoring impaired brain DHA levels due to previous (maternal) dietary n-3 deficiency, than exposure to a diet containing the same amount of PL but added as an ingredient to regular size and uncoated lipid droplets (*Schipper et al. unpublished data*). In this latter study, however, the PL used to prepare the experimental diets were derived from egg lipids rather than bovine MFGM and the potential influence of PL-sourcing remains to be investigated. Nevertheless, these studies together suggest that next to molecular structure, also the supramolecular structure of lipids in nutrition may be an additional factor capable of influencing brain n-3 LCPUFA accumulation early in life. More research is needed in this area.

Building blocks: other (bioactive) dietary lipids influence brain lipid composition

A diet with large, PL coated lipid droplets resulted in higher cognitive function and lower anxiety like behaviour in mice (describe in **Chapter 4 and 6**). In order to produce the specific PL coating in the Concept diet, the bovine MFGM ingredient was added during processing. MFGM is rich in complex lipids including PL, sphingolipids, gangliosides, and cholesterol (73, 75-79), which are present in human milk as well but not in standard IMF. Like LCPUFA's, these complex lipids can also be found in neuronal membranes where they are involved in structural and functional membrane properties (80-83). It is widely accepted that these membrane

lipids play an important role in brain development, affecting a variety of processes including neurotransmission, neurogenesis, synaptogenesis, modulating synaptic transmission, cell proliferation, and neuronal differentiation and myelination (84, 85). Therefore, higher dietary supplementation of these lipids may contribute to brain development and function. Moreover, some of these complex dietary lipids contain (conditionally) essential nutrients such as sialic acid, a component in gangliosides, and choline, present in phosphatidylcholine and sphingomyelin, that are required for neurodevelopment (86-88). These complex dietary lipids may be absorbed by the intestine as whole (89) or as individual essential components after digestion, and transported to the brain where they are incorporated in neuronal membranes or used again as a precursor for de novo biosynthesis of brain lipids (90). A higher dietary supply of these preformed complex lipids may therefore increase the presence of these lipids in the neuronal membrane. Indeed, breastfed infants were shown to have a higher brain ganglioside and sialic acid concentration than infants that had been fed a standard formula in which the content of these components is low (91). In rats, early life supplementation with dietary gangliosides increased brain ganglioside content (92, 93) which was associated with higher neuroplasticity (94) and higher scores in cognitive tests (95). Although there is no information available on dietary cholesterol and brain cholesterol levels in human infants, increased brain cholesterol levels were found after early life dietary supply of cholesterol in piglets (96) and rodents (97), where it was suggested to contribute to myelination. Together, these observations suggest that exogenous supply of these complex lipids might be making a significant impact on the neurological development of the human brain. As described in **Chapter 4**, we did not detect higher brain PL content in adult mice that had been previously fed a diet with large PL coated lipid droplets. The total concentration of PL that was present in the diet was very low and brain PL content was determined in adulthood, 8 weeks after termination of the diet. It remains to be investigated whether the low concentration of dietary PL or some of the other preformed bioactive lipids present in Concept diet used in **Chapter 4 and 6** are effective in increasing the abundance of brain PL and or other bioactive lipids such as cholesterol and gangliosides at earlier stages of development.

Neurodevelopmental cues: PUFA

Other than being accreted from plasma and used as a structural component of neuronal membranes, circulating fatty acids can be used as signalling molecule, to inform the brain about the body's energy and nutrient status. Neurons in the arcuate nucleus of the hypothalamus (ARH) constantly monitor circulating hormones and nutrients that indicate the body's energy and nutrient state and respond to alterations in these factors by adjusting food intake behaviour, energy expenditure, and the neuroendocrine/autonomic outflow in order to maintain a homeostatic balance. In particular the adipocyte derived hormone leptin is well known for its role as important regulator of energy metabolism (98) but hypothalamic neurons also respond to fatty acids directly. Although the exact neuronal mechanisms involved in fatty acid sensing and molecular processing are not well understood (99-103), fatty acids are known to modulate hypothalamic neuron activity and neuropeptide

expression directly or indirectly via astrocyte FA metabolism (103-106) with n-3 LCPUFA's exerting an anorexigenic effect (107-109). The hypothalamic responses and or effects on energy balance to fatty acids appear to be specific for the fatty acid type and saturation level (110), omega 3 and 6 fatty acid may evoke opposite responses. For example, centrally administered DHA increased hypothalamic POMC expression and reduced food intake (109), thereby acting as satiety factor similar to the actions of leptin, while ARA inhibits hypothalamic leptin signalling (111). Key to proper integration and response to peripheral cues is however the axonal connectivity of hypothalamic nuclei, which is established in early life only. Interestingly, the same hormones that function as hunger and satiety factors in the mature hypothalamus act as specific neurodevelopmental cue and mediate the structural development (axon growth and connectivity) of the hypothalamic circuitry during this period. The specific neurodevelopmental role is restricted to early life periods only and has been demonstrated so far for several hormones including leptin (112), ghrelin (113, 114), and insulin (115). By modulating the levels of these hormones in the circulation during perinatal life, the nutrient and energy status contributes to shaping the structural formation of the hypothalamic circuitry and thereby establish the basic setpoint for energy balance regulation throughout life (116). In **Chapter 3** we described that postnatal diets with a lower n-6/n-3 ratio reduced the outgrowth of axons from the ARH to the paraventricular nucleus of the hypothalamus (PVH) in the hypothalamus. These structural alterations were permanent and are likely to change the functioning of the hypothalamic circuitry for life, thereby programming mice for reduced body fat accumulation in adulthood. Indeed, in two other studies, it was shown that these diets protect mice against excessive body fat accumulation when challenged with WSD in adulthood (2, 117). Surprisingly however, the structural changes in the hypothalamic circuitry were not mediated by diet induced alterations in circulating leptin or ghrelin during the lactation phase, which is the specific period where these hormones are known to act as developmental cues. It was certain however, that the fatty acids in the circulation were changed by the diet, reflecting the dietary (milk) fatty acid composition. The developing hypothalamus was therefore exposed to (relatively) higher n-3 LCPUFA's including DHA and lower n-6 LCPUFAs such as ARA during circuit maturation. As the activity of (adult) hypothalamic neurons can be modulated by circulating fatty acid composition, it may be possible that, similar to hunger and satiety hormones, circulating fatty acids may be picked up by the developing hypothalamus as well and used as neurodevelopmental cues for circuitry formation. Alternatively, the modulatory effects of ARA on hypothalamic leptin signalling (111) may be interfering with normal hypothalamic development during the early postnatal phase. Additional experiments are needed to examine whether fatty acids indeed have this neurodevelopmental role, and what the individual contribution of n-3 or n- 6 fatty acids in hypothalamic programming is.

Peripheral organ/tissue-mediated effects of dietary lipids on brain development and function

The process of brain development is regulated by many factors including specific genes, growth factors, hormones, behaviour and environment. Next to the effects of absorbed dietary lipids and their component on brain development as described in the previous paragraphs, dietary lipids can influence brain development through modulating the development and function of peripheral organs and tissues that, in turn, secrete hormones and factors important for neurodevelopment. A good example of a peripheral hormone with a specific neurotrophic action early in life is the adipocyte derived satiety factor leptin. During the first 3 weeks of postnatal life, a distinctive surge is seen in circulating leptin levels in rodents (118). In this period only, leptin is a critical factor for axonal outgrowth of hypothalamic ARH neurons to neurons in the PVH (112). Both the timing and amplitude of the postnatal leptin surge are important for normal anatomic development of the hypothalamic circuitry (112, 119, 120), and are regulated by (maternal) dietary lipid quantity (121, 122) and total availability of nutrition during postnatal life (123). While Korotkova et al (124) reported that the perinatal maternal dietary FA composition altered postnatal leptin levels in rats, we did not observe such effect due to the postnatal maternal diets varying in n-6 n-3 fatty acid composition (**Chapter 3**). This suggests that the observed changes reported by Korotkova et al were mediated by the 10-day gestational intervention rather than the postnatal intervention and that postnatal dietary fatty acid composition does not affect the normal leptin surge. Nevertheless, modulation of postnatal leptin surge may provide a mechanism by which other aspects of postnatal lipid quality, which also includes the supramolecular structure of dietary lipids, may exert their effects on hypothalamic development and thereby contribute to the neural control of energy metabolism later in life. Preliminary data from a rat study showed that exposure to a diet a with large, PL coated lipid droplets from PN 16 onwards, similar to the diet used in **Chapter 4** and 6, reduced the circulating leptin levels at postnatal (PN) day 21 (*Schipper et al, unpublished data*). The hypothalamic circuitry in rodents is not completely developed until the end of the 4th week of life (112, 125). Until PN 28, leptin does not acquire its anorectic effect as seen in adulthood (126) and it is believed that a gradual developmental shift takes place during the 3rd and 4th week of life in the action of leptin from neurotrophic factor to anorexigenic signal (127). This suggests that diet induced changes in leptin levels during the 3rd and 4th week of life are also capable of modifying the last steps in the formation of the HYP circuitry and affect function of this system for life. This idea is further supported by the observation that animals fed the Concept diet showed slightly increased satiety after peripheral leptin administration during adolescence and adulthood (*Schipper, unpublished data*) and a reduced body fat accumulation after adult exposure to high fat diets (**Intermezzo** and (128, 129)). Both may be indicative for a different functioning of the hypothalamic circuitry in adulthood by hypothalamic programming.

Other postprandial hormones may also contribute to brain development. The diet mimicking the supramolecular lipid structure of lipids in mammalian milk increased cognitive function in mice (**Chapter 4 and 6**), and the effects were observed specifically in behaviour tests that involved novelty exposure. Learning and memory performance can be increased by Locus Coeruleus (LC) noradrenergic (NA) stimulation of the prefrontal cortex and hippocampus,

which is typically activated by arousal during novelty exposure (130-132). It is possible that the concept diet in particular stimulated the development of this LC-NA system early in life which may impact the response during novelty arousal at later life stages. In line with this, it has been reported that lipid (energy) content of the diet can module the enhancing effect of arousal on memory performance in rats (133) and in human adults, bovine milk derived PL interact with the stress response to increase cognitive function (134). The different arousal levels due to the supramolecular structure of the lipids are likely mediated by different postprandial release of satiety hormones after ingestion. In human adults, the same Concept diet as used in **Chapter 4 and 6** resulted in prolonged release of the small intestine derived satiety hormone cholecystokinin (CCK) (74). CCK is known to simulate LC activity (135) and could thereby augment arousal, facilitating learning and memory processes (136). Exposure to the Concept diet following normal lactation may have a long term influence on novelty induced arousal or stress response as the development of the LC neurons and the projections to their target area's continue to develop during postnatal life in rodents (137-139) and NA may act as an important neurotrophic factor at its target area's in this developmental period (140-143).

On the longer term, differences in growth and organ specific development induced by dietary lipids may also result affect brain development and or function. Recent work suggests that skeletal development is a determinant of brain development, neuronal structure and behavioural function. Bone tissue secretes the hormone osteocalcin (OCN), which crosses the blood brain barrier to promote synthesis of several neurotransmitters including serotonin, dopamine, NA and reduces GABA during postnatal life (144). Mice lacking OCN show increased anxiety, depression and impaired learning and memory (144). This suggests that OCN is required for normal structural and functional brain development, and altered OCN secretion from skeletal tissue can modulate brain development. OCN synthesis is dependent on the maturity of the osteoblast and on the lipid soluble factors vitamin D and K (145-147). A diet with a supramolecular structure of lipid droplets mimicking mammalian milk lipids increased growth rate and bone development in young mice and increased adult cognitive function (**Chapter 6**). The increased bone development reflected as higher longitudinal growth and thickness (and possibly a higher absorption of dietary vitamin D or K due to this mammalian milk like supramolecular structure (148, 149)), may have resulted in higher osteocalcin synthesis, contributing to the reduced anxiety and higher cognitive function that were observed in these mice. Also the generally accepted link between early life dietary supplementation with n-3 LCPUFA (and or dietary low n-6 LA exposure) and cognitive and behavioural function could be mediated, in part, by enhanced bone development. High levels of ARA-derived prostaglandin 2 were shown to impair bone formation (150) and omega-3 fatty acid supplementation in young rats and mice amplified bone formation and OCN secretion (151-153).

Critical windows of opportunity

The examples described above show that dietary lipid quality may influence developmental processes that take place only during restricted time frames over the course of brain development. Effects of nutritional environment on these developmental processes are likely limited to certain specific maturation stages, and once developmental milestones are reached, the sensitivity to further nutritional modulation on that structure/system becomes obscured. In addition to maturation stage, the impact of dietary lipid quality on brain development strongly depends on the individual's health status and other (environmental) factors that influence the nutritional requirements and or the nutrient availability for the developing brain. Together with developmental stage, these factors form the so-called window of opportunity for nutritional intervention. For instance, the accumulation of DHA in the foetal brain rapidly increases during the last stages of pregnancy (154, 155). Infants that are born prematurely miss out on this final DHA accumulation in utero and have lower DHA status at term age than same gestational age infants born term (156). Preterm infants are at high risk for neurodevelopmental problems (157-159) which may be related to insufficient levels of DHA in the brain compared to term infants (8, 160, 161). The low n-3 LCPUFA status in preterm infants increases the window of opportunity for nutritional intervention with n-3 LCPUFA's. Indeed, (maternal) dietary n-3 LCPUFA supplementation seems to benefit neurodevelopmental outcomes in preterm infants more than healthy term infants (160, 162). Interestingly, consumption of human milk improves preterm brain development (i.e. increased brain growth, higher cognitive function) (163, 164). Moreover, the DHA levels in milk of mothers giving birth to preterm infants are higher than those who deliver at term (165) suggesting a natural compensatory mechanism to support the increased DHA requirements for the preterm infant. On the other hand, for both term and preterm infants, the window of opportunity for beneficial effects of DHA supplementation on brain development may be reduced by excessive levels of LA in HM. In Western-industrialized societies, there has been a marked increase in the intake of LA over the last decades, which is largely caused by the increased use of vegetable oils rich in LA. At the same time, the consumption of food products that contain n-3 LCPUFA's, such as fish, are typically low (166-168). The contemporary increase in dietary LA intake is also reflected in human milk with increased LA content in milk of women from Europe, Australia, and Northern America (48, 51). Because of the inhibitory effect of a high dietary supply of PUFAs on DHA synthesis (169) and the competitive interaction between n-6 and n-3 FA's in both LCPUFA synthesis (48) and incorporation in neuronal membranes, high LA levels in milk could lead to excessive n-6 LCPUFA and reduced DHA and other n-3 LCPUFA accumulation in the brain during development. Dietary DHA supplementation on high LA background did not overcome this excessive accumulation of n-6 LCPUFAs and low levels of n-3 LCPUFA in the developing piglet brain (17). In addition, increasing the total amount of dietary PUFA It is proposed that actively lowering LA intake in humans could reduce the dietary needs for n-3 LCPUFAs up to a tenth of the current intake, in order to meet adequate tissue n-3 LCPUFA status (46).

Factors in the environment may also modulate health status and or nutrient availability to the developing brain and can thereby interact with nutritional programming by dietary lipid quality. As described in **Chapter 5** individual housing in rodents is an environmental factor that can causes psychosocial and metabolic stress and thereby changes the availability, use and requirements of energy for developing organs including brain centres that are involved in the regulation of food intake. This may increase the risk for excessive body fat accumulation and the development of other metabolic derangements following a Western Style Diet. The preventive effect of a postnatal diet with more HM like supramolecular lipid structure on adult excessive body fat accumulation by WSD exposure was lower in individually housed mice compared to socially housed mice (**Intermezzo**). This suggests that the individual housing *decreased* the window of opportunity for beneficial nutritional programming of (adult) energy balance regulation. Regardless of early life programming diet, there was a higher adult body fat accumulation seen in individually housed compared to socially housed mice after WSD exposure. The individual housing therefore also *increased* the window of opportunity for adult diet-induced obesity. Individual housing, however did not appear to change the window of opportunity for a diet with altered supramolecular lipid structure to improve brain function (i.e. cognitive function, anxiety, social interest, **Chapter 6**).
It is worth mentioning that there are a number of additional environmental and experimental factors that may apply to the preclinical models described in this thesis and that could, in itself, affect offspring brain development and thereby potentially affect the window of opportunity for nutritional intervention. These factors include potential diet induced changes in maternal behaviour, i.e. maternal care and /or milk yield or milk composition (170, 171), maternal parity affecting maternal and offspring fatty acid status (172, 173), the number of siblings in the litter influencing dietary quantity (174, 175)and sex ratio of siblings in the litter affecting maternal care (176). In addition, climate conditions in the laboratory such as environmental temperature, noise, lighting can affect rodent physiology and behaviour, and interact with brain development as well (177). Although these factors were controlled for as much as possible in our current studies, it could be of relevance to investigate whether some of these factors could be used to modify the window of opportunity for nutritional intervention. Together, these examples show that the window of opportunity for brain developmental and physiological modulation by dietary lipid quality is influenced by developmental stage, health status and other environmental conditions.

Translation of the observed results to human situation

Our studies (**Chapter 2-4, 6**) show that the lipid quality of the postnatal diet influences various aspects of brain development and (long term) brain functioning in mice models. The purpose of these studies was to investigate the potential mechanism by which early life dietary lipid quality may contribute to brain development and function in human infants. Whereas all mammals follow roughly the same steps in brain maturation, a direct transfer of all described results to the human situation warrants caution as there are differences between humans and rodents in rates, duration, and timing of molecular and anatomical

developmental events and milestones and the emergence of age dependent behaviours. Rodents have a shorter life span and maturation of organs and systems is accelerated when compared with humans. In general, the stage of maturation of the rodent brain at the time of birth is considered "immature" when compared with that of humans at normal term birth, whereas at weaning (i.e. postnatal day 21), rodents are at a more mature stage than weaning age of human infants (i.e. 6 months) (see for reviews of key developmental processes in brain across comparable ages in humans and rodents (178, 179)). In the following section, we describe the separately the translatability of the result obtained in the various chapters of the current thesis describing different aspects of brain development and nutritional interventions.

Translating from mice to humans: effects of PUFA diet starting at PN day 2 on brain fatty acid accumulation and fatty acid composition in neuronal membranes

The study in **Chapter 2** in this thesis shows that in mice, the dietary supply of higher n-3 LCPUFAs or reduced linoleic acid to offspring during the lactation period increases brain n-3 LCPUFA accumulation (including DHA) in the suckling offspring during lactation. Higher n-3 LCPUFA accumulation in the brain is regarded as beneficial as sufficient n-3 LCPUFA's in neuronal membranes are critical for various processes during neurodevelopment and function, and low levels are associated with impairments in neuronal function (see above). After birth, mammals rely on LCPUFA's in maternal milk as an important source of brain building blocks. In mice, the average duration of lactation is 3 weeks while for humans 6 months of exclusive breastfeeding is recommended. Despite the differences between species in developmental stage of specific brain structures during perinatal life, the general pattern of n-6 and n-3 LCPUFA accumulation in the brain during pregnancy and postnatal development appears to be quite similar for human infants and rodent pups. Towards the end of pregnancy, the relative accumulation rate of n-3 LCPUFA DHA in the brain is increasing fast and in the final stages of pregnancy it exceeds that of ARA in both humans (154, 155) and rodents (180). After birth, ARA accumulation reaches a plateau while DHA continues to increase in humans infants (up to adolescence (181)) (8) and rodents (180) which can be modulated by the level of preformed n-3 LCPUFA's in the milk in both species (**Chapter 2**, (161, 182)). The higher DHA accumulation observed in mice offspring brain by maternal DHA supplementation during lactation may therefore directly translate to the human the human situation. In **Chapter 2** we also describe that a reduction of LA (the essential n-6 fatty acid and precursor of n-6 LCPFA) in the maternal diet resulted in higher n-3 LCPUFA accumulation in the offspring brain. In contrast to what was observed with maternal n-3 supplementation, there was no higher level of preformed n-3 LCPUFA's in the milk of dams in this low-LA group. As the other source for offspring circulating DHA is endogenously synthesized n-3 LCPUFA from the dietary n-3 precursor ALA in offspring tissues (48), it was concluded that the low-LA supply in maternal milk stimulated the endogenous synthesis of DHA in the mouse pups. It is generally accepted that rodents have a higher endogenous LCPUFA enzymatic capacity than humans. Especially in human infants, the DHA synthesis capacity is low and thought to be insufficient to support the high requirements for the developing brain after birth (183,

184). It is generally believed that in rodents the synthesis capacity for DHA is much higher than in humans, although recent studies challenge this view (185). We believe that despite the species difference, our results described in **Chapter 2** are of relevance to brain LCPUFA accumulation in human infants. In human infants, LCPUFA synthesis capacity is low, but not completely absent (47, 186). This means that a reduced supply of dietary LA to human infants will also reduce the relative endogenous synthesis of n-6 LCPUFA in infants, thereby stimulating n-3 LCPUFA (DHA) synthesis and availability for accumulation in the brain. In addition, reduced levels of LA in the maternal diet also directly target LCPUFA synthesis in the lactating mother.

Human DHA synthesis may be more efficient in women of child bearing age than in men or in infants (187, 188). Besides diet, the fatty acid composition of human milk further depends on the circulating fatty acid pool and endogenous synthesis in of LCPUFA's in the mammary gland, which are again influenced by maternal dietary fatty acids (189). It may be hypothesized that a reduced LA supply to the mother may stimulate maternal endogenous n-3 LCPUFA synthesis and thereby increase preformed n-3 LCPUFA content in milk. In our mouse study, however, we did not find such an effect, which may be explained by the overall low content of total LA, and low PUFA content in general in both the low-LA and control diets that were used in this study compared to those in normal rodent chow (Reeves 1993). In humans, the intake of LA worldwide has dramatically increased over the last decades due to industrial food processing which has also resulted in an increased LA content in human milk (51, 169). The high levels of LA in maternal milk stimulate the synthesis and brain accumulation of n-6 LCPUFA over n-3 LCPUFAs, and are negatively associated with (long term) neurodevelopmental outcomes in infant (190, 191). Moreover, high milk LA may limit the effectively of nutritional supplementation with preformed DHA for brain development. Although human trials cannot be conducted on this specific topic for ethical reasons, it was shown in piglets that DHA supplementation on high LA background could not overcome the disadvantageous high n-6 LCPUFA and low EPA accumulation in the brain that were caused by excessive LA consumption (17). This may be explained not only by the competitive interaction between n-6 and n-3 PUFA's for conversion to longer chain PUFA's and uptake in brain tissue, but also by the fact that high dietary supply of (n-6) PUFA's inhibits the rate of (n-3) LCPUFA synthesis (48). Lowering dietary LA in humans may even strongly reduce the dietary requirements for n-3 LCPUFA's for normal development (46). A reduction in maternal LA supply during lactation (and gestation) is therefore a relevant target to improve infant brain n-3 LCPUFA accumulation.

Whereas we propose that brain PUFA accumulation following maternal dietary LA reduction and or DHA supplementation during the lactation phase in mice can be used to model the consequences of human maternal dietary PUFA interventions on infant brain fatty acid accumulation after birth, a direct translation of the functional relevance of these interventions during lactation in mice to humans may be more difficult. In general, the staging of brain development in mice during the first postnatal week translates to that of a human infant during the 3rd trimester (178, 179). For example, synaptogenesis is a process that is particularly sensitive to DHA levels in neuronal membranes (15, 192). In rodents the synaptogenesis primarily takes place during the first 3 weeks of postnatal life (178)

and therefore maternal n-3 LCPUFA supply during lactation could stimulate this process. In humans however, synaptogenesis commences at 20 weeks of gestation and continues, depending on the region, until about 2 years of age (193). To target synaptogenesis in its initial phase, maternal n-3 LCPUFA supplementation has to start during pregnancy. Therefore, it seems reasonable to assume that for these specific neurodevelopmental processes, the consequences of (maternal) dietary PUFA interventions during lactation in mice, as described in **Chapter 2**, may also correspond to a nutritional intervention in humans during the 3rd trimester. However, in the current thesis we did not further analyse the functional consequences of the altered brain membrane PUFA composition, nor did we investigate the effects of maternal dietary fatty acids on the transfer of n-3 and n-6 PUFA's across the placenta.

Translating from mice to humans: effect of PUFA diet starting at PN day 2 on hypothalamic development

In **Chapter 3**, it was shown that the same maternal diets that increasing the content of preformed n-3 LCPUFA's or reducing LA content in maternal milk by maternal dietary intervention altered the structural formation of the hypothalamic circuitry that is responsible for control of the balance between food intake and energy expenditure. Both diets resulted in a reduced density of orexigenic and anorexigenic ARH axonal projections to the PVH, which may influence system function. Although these anatomical alterations were difficult to interpret based on the current knowledge of orexigenic and anorexigenic pathways, they were associated with reduced body fat accumulation in adulthood and were therefore regarded as beneficial. Altered ARH neuronal axon formation (hypothalamic programming) might be a common phenomenon underlying metabolic programming and adult obesity risk (194). Dietary fatty acid composition is recognized as one of the dietary factors capable of metabolic programming (195). Early in life, infants exposed to HM with high n-6 /n-3 PUFA ratio show higher adipose tissue deposition (196). In previous preclinical studies it was found that mice exposed to diets with lowered LA content or increased n-3 LCPUFA content early in life were more resistant to excessive body fat accumulation by WSD in adulthood (2, 117), which may be caused by differences in energy balance regulation. The hypothalamic structural alterations were established within the first 4 weeks of life, during which the formation of the hypothalamic circuitry is known to take place in mice (112, 125). The development of the hypothalamic circuitry in human infants has not been studied due to ethical and methodological limitations. Human hypothalamic development is classically thought to take place mostly during the third trimester gestation (197, 198), which is based on what was known about hypothalamic circuit formation in non-human primates (199), but may need to be reconsidered (see below). As peripheral factors including dietary fatty acids and or other dietary lipid quality derived factors can influence outgrowth of ARH axons during the critical phase of circuit formation only, it seems reasonable to assume that for human hypothalamic circuit formation, the maternal dietary lipid quality during the 3rd trimester of gestation is a stronger determining factor than the lipid quality of the infant diet after birth. In non-human primates, a maternal HFD during pregnancy resulted

in alterations in the central melanocortin circuitry and reduced AgRP fiber density to PVH in the offspring hypothalamus (199, 200). In another study from the same group it was shown that these offspring showed catch-up growth during the postnatal period, leading to early-onset obesity (201) which together supports the notion of nutritional programming of the hypothalamic system occurring during pregnancy. Whereas in rodents the sensitive period for hypothalamic programming by dietary quality continues into the postnatal period due to the concurrent axonal development (116, 202) the timing of in particular the actual closure of the sensitive window for hypothalamic programing in primates including humans has not been defined. For non-human primates it was suggested that further refinement of the hypothalamic circuitry continues during the post-natal period as the ARH fibre density in adulthood was higher than that during late foetal life (199). In line with this, a very recent study showed, for the first time, that postnatal exposure to HFD also altered the AgRP fibre density in the ARH of non-human primates (203). Together, these observations suggest that in humans, the sensitive period during which dietary lipids can modulate offspring hypothalamic development starts during the 3rd trimester but may indeed extend into the early postnatal period as well. As the maternal dietary fatty acid composition passes through the placenta and transfers into her milk (204, 205), a maternal intervention with altered dietary FA composition during lactation in mice may correspond to a human maternal dietary intervention during gestation and lactation. Optimizing the supply of n-6 and n-3 LCPUFA's to the developing hypothalamus by such dietary interventions during gestation and lactation may be used as a strategy to prevent obesity and metabolic syndrome in humans as early and as long as possible.

Translating from mice to humans: effects of supramolecular structure of lipids in diet starting at PN 16 on cognitive function

Our studies in **Chapter 4 and 6** describe that the performance of mice in specific tasks was increased due to exposure to a diet with altered supramolecular structure of lipid droplets starting at PN 16. These effects indicate improvements in brain development and or functioning. It was hypothesized in **Chapter 4 and 6** that one of the underlying mechanisms that could explain the differences between diet groups in behavioural performance is a different activation or functioning of the Locus Coeruleus (LC) noradrenergic (NA) system. Cognitive functioning can be enhanced by LC-NA system activation, which takes place during novelty induced arousal. The LC-NA system is also responsive to circulating postprandial satiety hormones, that may be influenced by the diet with altered lipid structure (74). This may be especially relevant during postnatal development, when NA acts as a neurotrophic factor at the LC target areas in the brain (140-143). Alternatively, an increased or prolonged activation of the LC during critical periods of brain development could alter structural development of LC neurons and their projections. These projections mature during postnatal life in rodents (137-139). In humans, the pre and postnatal development of the LC-NA system is less well studied, however, differences in morphology and protein expression in LC neurons of post-mortem material of infants and foetuses that died of sudden (intrauterine) unexplained death syndrome suggest that the LC continues to mature

after birth in humans as well (206, 207). In the experiments described in **Chapter 4 and 6** the dietary intervention with large PL coated lipids started at PN 16, at which the stage of brain development for rodents may be roughly equivalent to that of 6 to 9 months of age in human infants (179). In humans, this age until approximately 5 years of age represents an important time for acquisition of fundamental cognitive skill in human infants, and therefore a period during which the brain and cognitive development may be sensitive to modulation of the nutritional environment (208). The World Health Organization recommends exclusive breastfeeding in humans until 6 months of age (209). Interestingly, in particular longer duration of breastfeeding (>6 months) is associated with improved infant cognitive function (210, 211). With the diets used in **Chapter 4 and 6** in this thesis, the exposure to one specific feature of breastfeeding (i.e. the supramolecular lipid structure) was prolonged by the dietary intervention starting directly after normal lactation. Based on these results, we hypothesize that the supramolecular structure of lipids in HM contributes to the beneficial effects of prolonged breastfeeding seen in human infants. Consequently, incorporating this more HM like supramolecular lipid structure in IMF products for infants up to at least 12 months of age, but preferable longer, to complement the period of normal breastfeeding, could contribute to cognitive development of formula fed infants.

Exposure to the altered dietary lipid structure improved cognitive function and reduced anxiety–like behaviour in mice that were reared individually from weaning onwards; these effects were associated with accelerated growth (**Chapter 6**). Both the reduced anxiety and the accelerated early postnatal growth can be seen as beneficial effects. Individual housing in rodents changes the energy balance regulation and, especially during periods of growth and development, provides a metabolic challenge as the increased thermogenesis may reduce the energy available to support normal growth and brain development (**Chapter 5**). The reduced growth rate observed in individual housed animals in our study could serve as a model for impaired postnatal growth in human infants. Childhood stunting and impaired postnatal growth are associated with long term impaired cognitive development and behavioural problems including anxiety and depression (212-215). The association between growth and neurocognitive outcome is in particularly seen in infants under challenged conditions i.e. infants born small for gestational age and/or infants born preterm (216). Exclusive breastfeeding was shown to reduce the risk of stunting (217). To this end, it would be interesting to investigate if (prolonged) exposure to the complex supramolecular lipid structure as seen in HM could potentially be used as part of a nutritional strategy to prevent or rescue impaired in brain and behavioural development in children suffering from faltering growth or born with low birth weight.

Chapter 5 shows that individual housing of rodents, especially during critical periods of growth and development, affects their energy balance regulation with (long term) implications for metabolic function, increasing the risk for metabolic disturbances (e.g. higher food intake and increased visceral fat deposition). Increased energy intake due to individual housing in rats and mice is in part explained by a compensatory mechanism to meet the higher energy demands for thermogenesis when housed individual under standard laboratory temperatures (218, 219). Increased energy intake in individually housed rodents may also be caused by different functioning of the hypothalamus (220-224) and higher brain area's

involved in the motivational and hedonic aspects of food intake (220, 225, 226) due to HPA activation as social deprivation may cause chronic stress and depression. In addition, stress induced alterations in glucocorticoid homeostasis affecting the CNS-adipose tissue axis can explain the increased energy storage (227-230). Whereas humans are not dependent on social thermoregulation, the stress and stress hormone mediated alterations in central regulation of food intake and body fat accumulation are likely to apply to humans as well (231, 232). Studies in humans have demonstrated that stress, disruption of social bonds and perceived isolation (loneliness) are associated with overeating and obesity (233-237), and specifically childhood stressful experiences including social adversity may contribute to later in life obesity development via mental and emotional dysregulation (238, 239)

Implications for human health

Human infants in our modern society may face many challenges during the first 1000 days that can affect brain development and are associated with higher risks for both mental and metabolic diseases. In our Western society, developing foetuses and new-borns are typically exposed to an imbalanced dietary supply of n-6 and n-3 PUFA's (i.e. excessive LA and insufficient n-3 LCPUFA) driven by maternal dietary habits affecting both placental transfer and HM composition (48, 51). This paradigm also applies to formula fed infants as the fatty acid composition of IMF has been based on human milk composition (240) analysed in a small set of Caucasian women in a Western industrialized society context of which the milk FA composition typically reflects the high n-6 content of the available diet (51, 241). These dietary habits may directly affect DHA accumulation in the developing brain and may explain the higher risk for neurological and psychiatric disorders such as depression and schizophrenia (242-244). In addition, a suboptimal n-3 LCPUFA status may compromise cognitive function. The WHO estimates that about one-third of the adult population suffers from a mental or neurological disorder (245) and children and adolescents are increasingly affected (246). As effective treatment of mental disorders is not only time consuming and expensive, but often simply not possible, prevention is key. A preventative rather than reactive approach in management of brain disorders, for example by ensuring the optimal balance of n-3 and n-6 PUFA's in early life could be beneficial. Our results describe that a (maternal) diet with either reduced LA content of increased n-3 LCPUFA content enhances the n-3 LCPUFA accumulation in neuronal membranes in the offspring (**Chapter 2**). Although potential beneficial effects of dietary n-3 supplementation have received a lot of attention in the literature, the effects of lowering n-6 supply is relatively understudied.

Our results also describe that exposure to either of these (maternal) dietary interventions leads to altered structural development of the hypothalamic circuit controlling energy intake and expenditure (**Chapter 3**). These data, together with previous results (2, 117), suggest that the diet-induced neuroanatomical alterations in the hypothalamic circuitry may increase the ability of the organism to cope with metabolic challenges during adulthood and thereby reduce the risk for obesity and metabolic disease. The prevalence of human obesity, metabolic syndrome (MetS) and related non-communicable diseases (NCDs) has increased

to epidemic proportions in industrialized as well as fast developing societies, putting a heavy burden on the health care system (247, 248). The increased LA content and lower n-3 LCPUFA in human milk has been hypothesized to be one of the factors contributing to the obesity epidemic (48, 51, 249, 250), but effects on specific aspects of brain development are limited. Our results suggest that ensuring an optimal balanced supply of n-3 and n-6 PUFA's to the developing brain during the first 1000 days may be used as a preventive strategy against mental disease, and may help to fight the obesity epidemic. Practically, this goal can be reached through more specific dietary advice to pregnant and lactating women regarding fatty acid intake. Although the importance of (maternal) dietary intake n-3 LCPUFA's for infant brain development is well known to the public, the potential adverse effects of LA, and therefore the importance of lowering levels of LA in (maternal) nutrition are not yet taken into account in current dietary recommendations. Lowering dietary intake of LA can be reached by replacing the consumption of standard vegetable oils and food items containing substantial LA quantities with alternative food products containing oils and food products lower in LA (251-253). These dietary advices are easily accessible, cheap and home based and therefore have the potential to reach a broad public. In line with this, the fatty acid composition in nutritional products for infants and young children should be adapted. The current regulations regarding LA content in IMF include a minimal and maximal allowance of 0.3 and 1.2 g /100kcal IMF respectively, an ALA allowance between 0.05 and 0.24 g /100kcal IMF, and a minimal LA:ALA ratio of 5 (240). As also proposed by others (e.g. (48, 241, 254)) the result described in the current thesis support the notion that the present upper levels of LA, maximal addition of ALA and the lower LA:ALA ratio limit in IMF should be reconsidered in addition to LC-PUFA addition recommendations.

In addition, the supramolecular structure of dietary lipids may be a promising target to explore in more detail. (Prolonged) breastfeeding is associated with a lower risk of rapid body weight gain (255-257) and better neurodevelopmental outcomes (210, 258, 259) and later in life mental and behavioual problems (260-262). Whereas possible confounding circumstances such as differences in parental education, health and food habits cannot be fully excluded in these human trials, we have shown in this thesis that the typical structural organization of dietary lipids in human milk, i.e. being present as large, PL-coated lipid droplets could contribute to some of these benefits (**Chapter 4 and 6**). Adapting the supramolecular structure in IMF for infants < 6 months but also in products for infants beyond 6 months may help to support beneficial long term outcomes to formula fed or mixed fed infants on brain development. Breastfed infants are reported to have better school performance during childhood compared to formula fed infants (263, 264), which extends into higher educational/academic performance during adolescence and even higher incomes in adulthood (264, 265). Better school performance builds human capital, health and well-being (266).

Interpretation and translation of the results obtained from animal experiments to the human situation should be done with caution. In **Chapter 5** we show that in rat and mouse studies, social housing conditions can have a strong impact on the metabolic phenotype of the animals. Outcomes of preclinical studies using rodents are often used for the design of human trials without taking into account potential effects of social and other environmental

conditions. We advocate for better awareness of this potential confounding factor in ongoing preclinical research and when evaluating the efficacy of drugs, diets or other interventions on metabolic health in reviews, systematic or otherwise.

Proposal for Future studies

The current thesis argues that adjustments in dietary lipid quality, being the fatty acid composition or the supramolecular structure of dietary lipids, influence development and long term brain function. It goes without saying that before any changes in recommendations for humans can be made, these preclinical findings should be further tested in randomized clinical trials in pregnant or lactating women and/or their infants. To be able to design proper trials in humans more information regarding the potential underlying mechanisms by which dietary lipid quality affects brain development would be helpful. Preclinical studies are indispensable for this, and could help to identify the right target population, further optimizing the nutritional intervention and exploring potential new benefits. Based on the results of the currents thesis a few recommendations for possibly interesting (pre)clinical studies can be made.

Improving dietary lipid quality by combining lipid composition and structure

In the current thesis, we studied the effects of postnatal dietary lipid quality on development of brain structure and membrane composition as well as (long term) function in mice. We found that reduced dietary n-6/n-3 PUFA ratio increased brain membrane n-3 LCPUFA content during lactation (**Chapter 2**). Preliminary data indicate that a diet with lipid structure may also increase brain membrane n-3 LCPUFA content albeit more modest (*Schipper et al. unpublished data*). As these two different aspects of dietary lipid quality appear to result in a similar shift in brain n-3 content, albeit potentially via different mechanisms, a logical next step would be to combine these two aspects of dietary lipid quality in a follow up study, e.g. large, PL coated lipid droplets with a TG core containing reduced LA levels and or increased DHA. This diet could potentially be provided during the post weaning period but also directly to the mouse dams during pregnancy and/or lactation (see below). Next to brain n-3 PUFA accumulation, possible synergistic or additive effects of the combination could be relevant for the development of specific brain structures and function other that studied in this thesis. Based on the literature, exposure to a diet with reduced LA content and/or increased DHA content may improve performance in a broader array of cognitive tasks and behaviours (9, 267). The modest effects of the postweaning diet with large PL coated lipid droplets on cognitive function (**Chapter 4 and 6**) may be more robust if combined with compositional improvements and/or earlier and longer exposure periods. We specifically showed in **Chapter 3** that adjustment in the dietary fatty acid composition during lactation was able to change the structural formation of the hypothalamic circuitry that controls energy balance regulation and that these effects were not mediated by a change in postnatal leptin levels. In contrast, preliminary findings using the diet with large, PL coated lipid droplets

indicate altered leptin levels at PN day 21 in rats (*Schipper et al. unpublished data*). Since the leptin surge can mediate structural development of the hypothalamic circuitry (268), we hypothesize that the diet with large, PL coated lipid droplets could potentially alter structural formation of the hypothalamic circuitry for energy balance regulation. Indeed, preliminary data using a leptin challenge suggests that animals fed the diet with large PL coated lipid droplets may be less susceptible to overeating after fasting and may be more sensitive to the inhibiting effects of exogenous leptin administration on food intake (*Schipper, unpublished data*). These arguments suggest that both diets may alter (structural) development of the hypothalamic circuitry, and a combination of the two may result in more robust effects on hypothalamic programming of long term (metabolic) regulation. Such an experiment could not only look at possible synergistic or additive effects but also provide more insight in critical windows of opportunity (i.e. (pre)pregnancy, lactation, gestation) during which lipid quality may be relevant for brain development and function. Maternal dietary PUFA crosses the placenta and in addition is translated into mothers milk FA composition and thus can be expected to reach the developing offspring brain during (pre)pregnancy, lactation and the post-weaning period. This is in contrast to the altered lipid structure being probably most relevant in the post lactation stage although it may contribute to altered lipid absorption in the dams as well (74). It is expected that the supramolecular structure in the new diet contributes maximally to offspring brain development from at least PN 16 onwards in mice, when they consume the diet themselves. At this time it is unclear how the supramolecular structure of lipids in maternal diet could potentially alter the quantity and/or quality of lipid supply to the offspring during gestation and lactation.

Maternal dietary lipid quality and maternal milk quality

In **Chapter 2** we showed that the fatty acid composition of the maternal diet during lactation affects maternal milk PUFA composition and, in turn, offspring brain PUFA composition. The maternal milk PUFA composition is therefore a relevant to improve, especially in our Western Society. In our study and in the work of others, improved milk fatty acid composition is often proposed to be the main factor that mediates brain benefits. However, maternal PUFA interventions may also contribute to infant brain development via other routes. For instance, maternal dietary PUFAs can be incorporated in virtually all biological membranes, including those in the mammary cells that form the MFGM encapsulating lipid droplets during milk secretion. Interestingly, the lipid composition of the mammary cell membrane is one of the factors that controls the size of the lipid droplets secreted (269). In dairy cows, it has been shown that the dietary (PUFA) composition can influence both size and membrane composition of the milk lipid globule (270-272). We have seen in the current thesis that large and PL coated lipid droplets in the diet following the normal lactation period can contribute to brain development and function (**Chapter 4 and 6**). Numerous reports in rodents and humans show that maternal dietary fatty acid intervention during gestation and or lactation can alter offspring brain development and function (see for a review e.g. (273)). In addition to fatty acid composition, it would be interesting to investigate possible changes in maternal milk lipid structure following PUFA intervention, and study if such changes could

contribute to altered neurodevelopmental outcomes. Theoretically, relatively small changes in maternal dietary fatty acid composition may alter not only the PUFA composition but also the structural characteristics of maternal milk lipids.

Early life diet and developmental and adult environmental challenges

All the studies described in this thesis were conducted in healthy mice born from from healthy dams. Moreover, all experimental and control diets including the western style diet met the minimal nutritional requirements for normal growth and development of rodents. In our design we limited handling procedures and optimized environmental conditions (other than offspring individual housing in **Chapter 6**) to minimize stress or disease risk. We anticipated that under these conditions offspring will show normal (optimal) brain development and that any changes in outcomes by early life nutritional interventions are relatively mild, if any. This holds especially true for dietary interventions that do not start until after lactation and involve no changes in nutrient composition, i.e. large PL coated lipid droplets studied in **Chapter 4 and 6**. In addition, the cognitive and behavioural tests as conducted in **Chapter 4 and 6** are designed to detect alterations in normal brain function; any improvements are rather difficult to reveal with these tests. Indeed, diet induced alterations on cognitive performance were mild, with improved performance observed only in two specific cognitive tests that rely on novelty exploration (T-maze and Novel Object Recognition, **Chapter 4 and 6**). Home cage behavior remained unaffected (**Chapter 4**) and although anxiety seemed to be reduced during adolescence, any effect of diet on anxiety and social interest in adulthood did not reach significance (**Chapter 6**). We hypothesize that the potential beneficial effects of these early life dietary lipid quality changes on brain development may be more clear when using preclinical models in which offspring brain development is compromised. These models could potentially also better mimic some of the many real life challenges that human infants and children face throughout their life course. Relevant examples of such models could include i) e.g uterine artery ligation (274), neonatal hypoxia and premature delivery (275) modeling neuronal damage seen in preterm or low birth weight infants or impaired fetal growth; ii) maternal exposure to excessive dietary LA (276) or ALA deficient diet (277) during pregnancy and or lactation impairing brain n-3 LCPUFA accumulation and modelling the excessive LA supply to developing fetuses and infants in the modern Western industrialized society; iii) maternal/early life stress (278) modelling infant adversities such as neglect and or abuse that may lead to later in life cognitive impairments; and/or iv) small litter rearing induced malprogramming of the hypothalamic circuitry (279) to mimic the effects of infant overfeeding as commonly seen in the Western society. For some of these models, sensitivity to dietary interventions to attenuate adverse effects on offspring brain and metabolic health has already been confirmed, e.g. (280-282). Depending on the timing of the "brain challenge or insult" and the nutritional intervention, potential beneficial effects of (maternal) dietary exposure to improved fatty acid composition and or a more mammalian milk like lipid structure could be investigated on the potential contribution to prevent, rescue or treat compromised brain development and function.

Changes in brain development and function induced by early life dietary lipid quality

may have a marked and persistent effect on brain function, including the capability of the individual in coping with environmental challenges in adult life. Exposing the mice to environmental challenges *in adulthood* may therefore reveal potential beneficial effects of early life dietary lipid quality that may stay unnoticed in a 'healthy' context. For instance, exposure to WSD during adulthood may impair cognitive and affective functions and affect anxiety-like behaviour (283-285). It would be interesting to also evaluate the effects of the early life diets with altered dietary lipid quality on brain function (**Chapter 4 and 6**) after adult exposure to WSD. Other relevant environmental challenges during adult life may be exposure to stressors, adverse nutritional or environmental conditions, pharmacological or experimental interventions that impair brain function. Lastly, another approach may be to increase the duration of the experiments and evaluate the potential effects of early life dietary lipid quality on the onset or the severity of age related cognitive decline. The prospective benefits of improved dietary lipid quality early in life for prevention, rescue or treatment of (environmentally induced) brain related impairments throughout the course of life is promising.

Concluding remarks

The experimental data presented in this thesis indicate that two different aspects of milk lipid quality, i.e. fatty acid composition and the supramolecular structure of lipids, can both modulate brain development and function when provided during critical phases of brain development in early in life. Our studies clearly showed that either decreasing the LA content or increasing DHA content in the milk supply shortly after birth improves accumulation of n3LCPUFA's in brain neuronal membranes. In addition, these paradigms result in a different structural development of the hypothalamic circuit responsible for the regulation of energy balance. In addition, we show that exposure to a diet with a supramolecular structure of lipids similar to those in HM (i.e. large, PL coated lipid droplets) in the post-weaning phase increased early life growth rate, reduced adolescent anxiety and improved (adult) cognitive performance (besides earlier reported metabolic programming benefits). These findings may help to explain, at least in part, some of the (long term) advantages that are typically observed in breastfed infants over formula fed infants regarding brain development and function as well as differences in metabolic health risk profiles later in life. Experiments using rats and mice are indispensable for studying these mechanisms and we showed that for instance housing conditions should be taken into account when evaluating the effects of diet on brain and metabolic health.

Greater knowledge of the mechanisms involved in nutritional modulation of brain development could result in improved dietary advice for pregnant and lactating women and could lead to improved design of human milk alternatives for infants that are not or no longer breastfed. The fact that the brain membrane composition, structure and function in early life set brain function for life makes dietary lipid quality an interesting target for intervention to support life-long brain health and prevent later life disease.

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